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[Continued on next page]

(54) Title: USE OF TETRAHYDROBIOPTERIN DERIVATIVES IN THE TREATMENT AND NUTRITION OF PATIENTS WITH AMINO ACID METABOLIC DISORDERS

(57) Abstract

[see original for abstract in English]

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Description

Use of Tetrahydrobiopterin Derivatives in the Treatment and Nutrition of Patients with Amino Acid Metabolic Disorders

This invention relates to the use of tetrahydrobiopterin derivatives according to Claim 1, a composition according to Claim 13, the use of tetrahydrobiopterin derivatives as nutritional supplements according to Claim 26, and a special food according to Claim 28, special low-phenylalanine foodstuffs according to Claim 40, as well as a diagnostic agent for the diagnosis of tetrahydrobiopterin-sensitive diseases that accompany amino acid metabolic disorders according to Claim 43.

Diseases resulting from amino acid metabolic disorders are relatively wide-spread as a set, and are generally genetically determined. Reduced activity of specific enzymes are a pathological physiological correlative, resulting in elevated or decreased concentrations of amino acids, neurotransmitters and messenger substances, as well as disorders in tolerance (protein intolerance) for certain albumin components in the food.

For purposes of this invention, the term "diseases resulting from amino acid metabolic disorders" means the following pathological physiological conditions:

Conditions with elevated phenylalanine or decreased tyrosine, serotonin or dopamine in bodily fluids, tissues or cells, particularly in the case of conditions with reduced phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase and NO synthase activity. These conditions may include the following diseases, but are not limited thereto: phenylketonuria, particularly mild phenylketonuria,

classic phenylketonuria; pigment disorders of the skin, particularly vitiligo; as well as conditions caused by decreased cellular availability of catecholamines, particularly orthostatic hypotension (Shy-Drager syndrome), muscle dystonia; as well as neurotransmitter disorders, particularly schizophrenia; conditions caused by reduced cellular availability of dopamine or serotonin as a result of tyrosine hydroxylase or tryptophan hydroxylase deficiencies, particularly Parkinson's disease, depressive disorders and dystonic movement disorders, conditions with reduced NO synthase activity, particularly endothelial dysfunctions, and deficient defense against infections.

One known amino acid metabolic disorder based on a deficient or depressed phenylalanine metabolism is hyperphenylalaninemia, caused by a phenylalanine hydroxylase deficiency. At least half the patients affected present with mild clinic phenotypes. The only treatment possible in the state of the art for most amino acid metabolic disorders, such as hyperphenylalaninemia, for example, is based on feeding patients diets using products that do not contain the amino acids affected by the special metabolic disorder, or contain only very limited quantities thereof.

Hyperphenylalaninemia was one of the first genetic disorders that could be treated. In most cases, hyperphenylalaninemia results from phenylalanine hydroxylase deficiency due to from mutations in the phenylalanine hydroxylase gene. The associated phenotypes range in severity from classic phenylketonuria (Online Mendelian Inheritance in Man No. 261600) (Online Mendelian Inheritance in Man No. 261600) to mild phenylketonuria and mild hyperphenylalaninemia. At least half of the affected patients suffer from one of the milder clinical phenotypes.

Patients with classic phenylketonuria and with mild phenylketonuria must follow lifelong low-protein diets, to prevent neurological sequelae and to ensure normal cognitive development; in contrast, patients with mild hyperphenylalaninemia do not require treatment, under certain circumstances. In connection with the very strict diet, there is a risk of diet-related deficiencies which pose a heavy burden for the patients and their families.

To date, there has been no effective causal treatment in the state of the art, so there is no possibility for patients other than complying with a strict diet if they do not wish to risk severe sequelae from the amino acid metabolic disorder and the related hyperphenylalaninemia, for example. The neurological sequelae include, for instance, irreversible damage to the nervous system and brain, and mental retardation, up to and including complete mental deficiency. Furthermore, kidney damage, liver damage and sensory organ damage have been reported.

For affected patients, this means – based on the example of hyperphenylalaninemia – that they must follow a diet low in phenylalanine. Since phenylalanine is an important component of protein, particularly in the animal world, it is naturally difficult to feed patients with amino acid metabolic disorders, without provoking undesired, toxic increases in phenylalanine. Furthermore, diet-related nutritional deficiencies may occur.

In the prior art, albumin hydrolysates were used for this purpose; they are produced from low-phenylalanine proteins by acid or alkaline hydrolysis.

Such products had a more than foul taste and were frequently intolerable for patients in the long term. In addition to these hydrolysates only foods selected according to strict dietary guidelines, mostly vegetarian in nature, could be considered for the affected patients.

In contrast, synthetic amino acid mixtures that do not contain amino acids affected by the metabolic disorder are already a great improvement over the previously-produced hydrolysates.

Phenylalanine-free products on this basis are known, for example, from US 5,393,532 and have been used since then as special foods for hyperphenylalaninemic and phenylketonuric patients.

Furthermore, the production of special foodstuffs based on casein glycomacropeptides in connection with amino acid mixtures is known from WO 98/08402 A1, for the feeding of patients without phenylalanine, for example, if needed.

In terms of taste, such amino acid mixtures fall far below the standard of usual foodstuffs.

In summary, it can be stated that a strict life-long compliant dietary plan tailored to a special amino acid metabolic disorder represents a heavy psychosocial burden, and other treatment methods have not been successful to date.

Starting from this prior art, this invention relates to making available substances that, on the one hand, can be used within the framework of therapeutic treatment of amino acid metabolic disorders, and on the other hand, can be used for the production of foodstuffs, particularly special dietetic foods for patients suffering from amino acid metabolic disorders.

The above task is solved by the use of tetrahydrobiopterin derivatives according to Claim 1, a composition according to Claim 13, the use of tetrahydrobiopterin derivatives as nutritional supplements according to Claim 26,

a special food according to Claim 28, as well as special low-phenylalanine foodstuffs according to Claim 40.

The invention also relates to making available a diagnostic tool for such amino acid metabolic disorders that can be favorably influenced by tetrahydrobiopterin derivatives.

This task is solved by a diagnostic tool according to Claim 43.

In particular, this invention relates to the use of at least one compound of the following general formula:

<graphic>

wherein R1 is selected from a group consisting of: H, OH, SH, F, Cl, Br, I, NH_2, $N(CH_3)_2$, $N(C_2H_5)_2$, $N(C_3H_7)_2$; and NH-acyl, wherein the acyl radical contains 1 to 32 carbon atoms, particularly CH_3O, preferably 9 to 32, and more preferably 9 to 20 carbon atoms;

wherein R2 is selected from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, O, and S;

wherein R3 is selected from a group consisting of: H, CH_3, and C_2H_5;

wherein R4 and R6 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, acetyl, and OX where X is a C1 to C32 acyl radical, particularly a C9 to C32 acyl radical, and preferably a C9 to C20 acyl radical;

wherein R5 is selected from a group consisting of: phenyl, CH_3, C_2H_5, C_3H_7, butyl, isobutyl, and t-butyl;

wherein R7 and R8 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, CH_3, COOH, CHO, and COOR9, wherein R9 is CH_3, C_2H_5, C_3H_7, or butyl;

wherein R10 is selected from a group consisting of: H, CH_3, and C_2H_5, and

-- represents an optional double bond; as well as

the pharmaceutically acceptable salts thereof;

for the manufacture of a medication to improve protein intolerance for the treatment of diseases secondary to an amino acid metabolic disorder.

Preferred embodiments of the use according to the invention are described below:

A compound is particularly suited for use according to the invention that is selected from the group consisting of: 5,6,7,8-tetrahydrobiopterin, sapropterin, particularly the hydrochloride or sulfate thereof, and a compound of the following structure:

<graphic>

(-)-(1'R,2'S,6R)-2-amino-6-(1',2'-dihydroxypropyl)-5,6,7,8-tetrahydro-4(3H)-pteridinone.

Particularly the dihydrochloride thereof, and/or

2-N-stearoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-decanoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-palmitoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-linoleoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin.

Hydrochlorides or sulfates can be used, in particular, as salts.

The above compounds can be considered, in particular, as medications for the treatment of the following diseases or amino acid metabolic disorders:

Conditions with elevated phenylalanine or decreased tyrosine in bodily fluids, tissues or cells, in particular conditions with reduced phenylalanine hydroxylase activity; phenylketonuria, particularly mild phenylketonuria, and classic phenylketonuria; pigment disorders of the skin, particularly vitiligo; conditions caused by reduced cellular availability of catecholamines, particularly orthostatic hypotension (Shy-Drager syndrome) and muscular dystonia; and neurotransmitter disorders, particularly schizophrenia.

A hydrochloride, particularly a dihydrochloride, is preferably used as the pharmaceutically acceptable salt.

Moreover, this invention is of special importance when at least one compound of the following general formula is used as a chaperone, particularly a chemical chaperone, or a so-called protein folding aid:

<graphic>

wherein R1 is selected from a group consisting of: H, OH, SH, F, Cl, Br, I, NH_2, $N(CH_3)_2$, $N(C_2H_5)_2$, $N(C_3H_7)_2$; and NH-acyl, wherein the acyl radical contains 1 to 32 carbon atoms, particularly CH_3O, preferably 9 to 32, and more preferably 9 to 20 carbon atoms;

wherein R2 is selected from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, O, and S;

wherein R3 is selected from a group consisting of: H, CH_3, and C_2H_5;

wherein R4 and R6 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, acetyl, and OX where X is a C1 to C32 acyl radical, particularly a C9 to C32 acyl radical, and preferably a C9 to C20 acyl radical;

wherein R5 is selected from a group consisting of: phenyl, CH_3, C_2H_5, C_3H_7, butyl, isobutyl, and t-butyl;

wherein R7 and R8 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, CH_3, COOH, CHO, and COOR9, wherein R9 is CH_3, C_2H_5, C_3H_7, or butyl;

wherein R10 is selected from a group consisting of: H, CH_3, and C_2H_5, and

-- represents an optional double bond; as well as

the pharmaceutically acceptable salts thereof.

When using a chaperone, it is preferred that the compound be selected from the group consisting of: 5,6,7,8-tetrahydrobiopterin, sapropterin, particularly the hydrochloride thereof, as well as a compound of the following structure:

<graphic>

(-)-(1'R,2'S,6R)-2-amino-6-(1',2'-dihydroxypropyl)-5,6,7,8-tetrahydro-4(3H)-pteridinone.

Particularly the dihydrochloride or sulfate thereof, and/or

2-N-stearoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-decanoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-palmitoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-linoleoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin.

The above compounds have proven outstandingly successful in reducing protein misfolding and thus lead to an improvement in enzyme activity, particularly in the case of structural abnormalities in enzymes that require tetrahydrobiopterin as a co-factor, e.g., in the case of defects in phenylalanine hydroxylase. Through this mechanism of action, they are preferably suited to the production of drugs suited to

the treatment of diseases attributable to structural abnormalities of the following enzymes: phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase or NO synthase.

Thus, the chaperones according to the invention are suited to the treatment of conditions

with elevated phenylalanine or reduced tyrosine, serotonin or dopamine in bodily fluids, tissues or cells, particularly in the case of conditions with decreased phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase and NO synthase activity.

This aspect of the present invention relates to the use of at least one compound of the following general formula as a neurotransmitter or messenger agent enhancer, particularly for catecholamines and/or serotonin and/or dopamine and/or nitrogen oxide (NO):

<graphic>

wherein R1 is selected from a group consisting of: H, OH, SH, F, Cl, Br, I, NH_2, $N(CH_3)_2$, $N(C_2H_5)_2$, $N(C_3H_7)_2$; and NH-acyl, wherein the acyl radical contains

1 to 32 carbon atoms, particularly CH_3O, preferably 9 to 32, and more preferably 9 to 20 carbon atoms;

wherein R2 is selected from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, O, and S;

wherein R3 is selected from a group consisting of: H, CH_3, and C_2H_5;

wherein R4 and R6 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, acetyl, and OX where X is a C1 to C32 acyl radical, particularly a C9 to C32 acyl radical, and preferably a C9 to C20 acyl radical;

wherein R5 is selected from a group consisting of: phenyl, CH_3, C_2H_5, C_3H_7, butyl, isobutyl, and t-butyl;

wherein R7 and R8 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, CH_3, COOH, CHO, and COOR9, wherein R9 is CH_3, C_2H_5, C_3H_7, or butyl;

wherein R10 is selected from a group consisting of: H, CH_3, and C_2H_5, and

-- represents an optional double bond; as well as

the pharmaceutically acceptable salts thereof.

A compound is preferred as a neurotransmitter or messenger agent enhancer that is selected from the group consisting of: 5.6.7.8-tetrahydrobiopterin, sapropterin, particularly the hydrochloride thereof, and a compound of the following structure:

<graphic>

(-)-(1'R,2'S,6R)-2-amino-6-(1',2'-dihydroxypropyl)-5,6,7,8-tetrahydro-4(3H)-pteridinone.

Particularly the dihydrochloride or sulfate thereof, and/or

2-N-stearoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-decanoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-palmitoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-linoleoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin.

This invention further relates to a composition containing at least one compound of the following general formula:

<graphic>

wherein R1 is selected from a group consisting of: H, OH, SH, F, Cl, Br, I, NH_2, $N(CH_3)_2$, $N(C_2H_5)_2$, $N(C_3H_7)_2$; and NH-acyl, wherein the acyl radical contains 1 to 32 carbon atoms, particularly CH_3O, preferably 9 to 32, and more preferably 9 to 20 carbon atoms;

wherein R2 is selected from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, O, and S;

wherein R3 is selected from a group consisting of: H, CH_3, and C_2H_5;

wherein R4 and R6 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, acetyl, and OX where X is a C1 to C32 acyl radical,

particularly a C9 to C32 acyl radical, and preferably a C9 to C20 acyl radical;

wherein R5 is selected from a group consisting of: phenyl, CH_3, C_2H_5, C_3H_7, butyl, isobutyl, and t-butyl;

wherein R7 and R8 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, CH_3, COOH, CHO, and COOR9, wherein R9 is CH_3, C_2H_5, C_3H_7, or butyl;

wherein R10 is selected from a group consisting of: H, CH_3, and C_2H_5, and

-- represents an optional double bond; as well as

---the pharmaceutically acceptable salts thereof; as well as

containing at least one amino acid that is selected from the group consisting of the essential amino acids: isoleucine, leucine, lysine, methionine, threonine, tryptophan, valine, and histidine; as well as the non-essential amino acids, particularly alanine, arginine, asparaginic acid, asparagine, cysteine, especially acetylcysteine, glutamic acid, glutamine, glycine, proline, serine and tyrosine.

A preferred composition is characterized in that it contains the essential amino acids selected from the group consisting of: isoleucine, leucine, lysine, methionine, threonine, tryptophan, valine, histidine and, in addition, at least one of the amino acids: alanine, arginine, asparaginic acid, asparagine, cysteine, particularly acetylcysteine, glutamic acid, glutamine, glycine, proline, serine and tyrosine.

It is further preferred that the composition according to the invention contain additional carbohydrates, particularly glucose, and/or vitamins.

Preferably, the composition according to the invention can be formulated as a preparation to be administered orally or intravenously.

The preparation can be formulated as a powder, tablet, capsule, coated tablet, drops or for topical use, particularly salves, as well as a solution for intravenous administration.

Of course, such preparations can be formed as pharmaceutical compositions with the usual galenic pharmaceutical adjuvants as needed.

The composition according to the invention can, however, also be formulated as a dietary supplement with adjuvants common in foodstuff technology, particularly emulsifiers, preferably lecithin or choline.

Furthermore, it is preferred that the composition according to the invention contain yet other minerals and/or electrolytes that are selected from: mineral salts; saline salts; sea salts; trace elements, particularly selenium, manganese, copper, zinc, molybdenum, iodine, and chromium; alkali ions, particularly lithium, sodium and potassium; earth alkali ions, particularly magnesium and calcium; and iron.

Within the framework of a dietary foodstuff for patients with hyperphenylalaninemia, the composition according to the invention can even contain additional phenylalanine without risking a toxic accumulation of phenylalanine in the serum, cerebrospinal fluid and/or brain.

It is further preferred that the composition also contain L-carnitine and/or myoinositol and/or choline.

Furthermore, it may be useful for the composition according to the invention to contain antioxidants common in foodstuff technology, particularly Vitamin C, thus avoiding the oxidative decomposition of tetrahydrobiopterin derivatives at least to a large extent, thereby improving the storage stability of the composition.

A composition is preferred with a compound in which the compound is selected from the group consisting of: 5,6,7,8-tetrahydrobiopterin, sapropterin, particularly the hydrochloride thereof, as well as a compound of the following structure:

<graphic>

(-)-(1'R,2'S,6R)-2-amino-6-(1',2'-dihydroxypropyl)-5,6,7,8-tetrahydro-4(3H)-pteridinone.

Particularly the dihydrochloride or sulfate thereof, and/or

2-N-stearoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-decanoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-palmitoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-linoleoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin.

This invention is of particular importance in the production of dietary supplements suited to providing patients suffering from amino acid metabolic disorders with a largely normal diet despite their disease.

In particular, the invention relates to the use of at least one compound of the following general formula:

<graphic>

wherein R1 is selected from a group consisting of: H, OH, SH, F, Cl, Br, I, NH_2, $N(CH_3)_2$, $N(C_2H_5)_2$, $N(C_3H_7)_2$; and NH-acyl, wherein the acyl radical contains 1 to 32 carbon atoms, particularly CH_3O, preferably 9 to 32, and more preferably 9 to 20 carbon atoms;

wherein R2 is selected from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, O, and S;

wherein R3 is selected from a group consisting of: H, CH_3, and C_2H_5;

wherein R4 and R6 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, acetyl, and OX where X is a C1 to C32 acyl radical, particularly a C9 to C32 acyl radical, and preferably a C9 to C20 acyl radical;

wherein R5 is selected from a group consisting of: phenyl, CH_3, C_2H_5, C_3H_7, butyl, isobutyl, and t-butyl;

wherein R7 and R8 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, CH_3, COOH, CHO, and COOR9, wherein R9 is CH_3, C_2H_5, C_3H_7, or butyl;

wherein R10 is selected from a group consisting of: H, CH_3, and C_2H_5, and

-- represents an optional double bond; as well as the pharmaceutically acceptable salts thereof;

as a dietary supplement.

As a dietary supplement for the above-mentioned group of patients, such a compound is particularly suited, especially when selected from the group consisting of: 5,6,7,8-tetrahydrobiopterin, sapropterin, particularly the hydrochloride thereof, as well as a compound of the following structure:

<graphic>

(-)-(1'R,2'S,6R)-2-amino-6-(1',2'-dihydroxypropyl)-5,6,7,8-tetrahydro-4(3H)-pteridinone.

Particularly the dihydrochloride or sulfate thereof, and/or

2-N-stearoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-decanoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-palmitoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-linoleoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin.

This invention is of outstanding importance in the manufacture of a special food based on essentially phenylalanine-free mixtures of amino acids with which particularly patients with hyperphenylalaninemia can be fed optimally.

Such special foods contain at least one compound with the following general formula:

<graphic>

wherein R1 is selected from a group consisting of: H, OH, SH, F, Cl, Br, I, NH_2, $N(CH_3)_2$, $N(C_2H_5)_2$, $N(C_3H_7)_2$; and NH-acyl, wherein the acyl radical contains 1 to 32 carbon atoms, particularly CH_3O, preferably 9 to 32, and more preferably 9 to 20 carbon atoms;

wherein R2 is selected from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, O, and S;

wherein R3 is selected from a group consisting of: H, CH_3, and C_2H_5;

wherein R4 and R6 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, acetyl, and OX where X is a C1 to C32 acyl radical, particularly a C9 to C32 acyl radical, and preferably a C9 to C20 acyl radical;

wherein R5 is selected from a group consisting of: phenyl, CH_3, C_2H_5, C_3H_7, butyl, isobutyl, and t-butyl;

wherein R7 and R8 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, CH_3, COOH, CHO, and COOR9, wherein R9 is CH_3, C_2H_5, C_3H_7, or butyl;

wherein R10 is selected from a group consisting of: H, CH_3, and C_2H_5, and

-- represents an optional double bond; as well as

the salts thereof acceptable in foodstuffs technology.

As a special food for hyperphenylalaninemic patients, such a product is particularly suited that contains at least one compound selected from the group consisting of: 5,6,7,8-tetrahydrobiopterin, sapropterin, particularly the hydrochloride thereof, as well as a compound of the following structure:

<graphic>

(-)-(1'R,2'S,6R)-2-amino-6-(1',2'-dihydroxypropyl)-5,6,7,8-tetrahydro-4(3H)-pteridinone.

Particularly the dihydrochloride or sulfate thereof, and/or

2-N-stearoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-decanoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-palmitoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-linoleoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin.

To ensure a complete nutritional offering, it is preferred that the special food according to the invention contain additional carbohydrates, particularly glucose, maltodextrin, starches and/or fats, such as fish oil, particularly salmon oil, herring oil, mackerel oil, or tuna fish oil.

It is particularly preferred that the special food be hypoallergenic and/or essentially gluten free.

Since most amino acid metabolic disorders are genetically inherited diseases, patients must be provided with the correct diet from birth onward. It is therefore a particular advantage that the special food according to this invention can be formulated as infant formula, particularly as a milk substitute for nursing infants as well as older children and adults.

Such a milk substitute for nursing infants has an additional fat content, present particularly as 90% triglycerides and 10% mono- and diglycerides.

For easier preparation and to increase the storage stability, the special food can be made available as a powder, particularly a freeze-dried product.

Additionally, it is preferred that the special food according to the invention also be provided with fatty acid supplements, particularly unsaturated fatty acids, preferably omega 3 fatty acids, especially alpha-linolenic acid, docosahexaenoic acid, eicosapentaenoic acid, or omega 6 fatty acids, in particular arachidonic acid, linoleic acid, or lineolenic acid; or oleic acid.

It is further preferred that the special food contain fish oil additives, particularly salmon, herring, mackerel or tuna fish oil.

Furthermore, the special food can have a fat component that includes plants oils, particularly safflower oils and/or soybean oil and/or coco oil.

A particularly preferred embodiment of the special food according to the present invention is formed as a milk drink mix, particularly a fruit-flavored or cocoa milk drink mix, based on its character as a milk substitute as well as specially for patients with amino acid metabolic, particularly hyperphenylalaninemia.

The present invention is of greatest importance in the feeding of patients with hyperphenylalaninemia: through the services of the inventor of this invention it is possible, for the first time, to provide such patients with a special low-phenylalanine food that is suited to increasing protein tolerance and the decomposition of phenylalanine, through the addition of tetrahydrobiopterin derivatives.

According to the present invention, such a special low-phenylalanine foodstuff contains a low-protein basic food as well as at least one compound of the following general formula:

<graphic>

wherein R1 is selected from a group consisting of: H, OH, SH, F, Cl, Br, I, NH_2, $N(CH_3)_2$, $N(C_2H_5)_2$, $N(C_3H_7)_2$; and NH-acyl, wherein the acyl radical contains 1 to 32 carbon atoms, particularly CH_3O, preferably 9 to 32, and more preferably 9 to 20 carbon atoms;

wherein R2 is selected from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, O, and S;

wherein R3 is selected from a group consisting of: H, CH_3, and C_2H_5;

wherein R4 and R6 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, acetyl, and OX where X is a C1 to C32 acyl radical, particularly a C9 to C32 acyl radical, and preferably a C9 to C20 acyl radical;

wherein R5 is selected from a group consisting of: phenyl, CH_3, C_2H_5, C_3H_7, butyl, isobutyl, and t-butyl;

wherein R7 and R8 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, CH_3, COOH, CHO, and COOR9, wherein R9 is CH_3, C_2H_5, C_3H_7, or butyl;

wherein R10 is selected from a group consisting of: H, CH_3, and C_2H_5, and

-- represents an optional double bond; as well as

the salts thereof acceptable in foodstuffs technology.

For the special low-phenylalanine foodstuff according to the invention, it is likewise preferred that a compound be used that is selected from the group consisting of: 5,6,7,8-tetrahydrobiopterin, sapropterin, particularly the hydrochloride or sulfate thereof, and a compound of the following structure:

<graphic>

(-)-(1'R,2'S,6R)-2-amino-6-(1',2'-dihydroxypropyl)-5,6,7,8-tetrahydro-4(3H)-pteridinone.

Particularly the dihydrochloride or sulfate thereof, and/or

2-N-stearoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-decanoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-palmitoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or

2-N-linoleoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin.

It is possible and preferred for the special low-phenylalanine foodstuff to be formulated as: convenience foods; pasta, particularly noodles; baked goods, particularly bread, cakes, and cookies; sweets, particularly chocolate, hard candies, and ice creams; drinks, particularly milk substitutes in the form of drink mixes, particularly fruit-flavored or chocolate drink mixes; and beer.

Hyperphenylalaninemic patients can thus, for the first time, eat significantly greater quantities of normal food – without putting themselves at risk due to their amino acid metabolic disorder – and without being ordered to use exclusively the foul-tasting products from the prior art.

As a result of the rapid onset of the effect of tetrahydrobiopterin derivatives, it is finally possible, within the framework of the present invention, to make available a diagnostic tool for detection of tetrahydrobiopterin sensitive diseases that contains at least one compound of the following general formula:

<graphic>

wherein R1 is selected from a group consisting of: H, OH, SH, F, Cl, Br, I, NH_2, $N(CH_3)_2$, $N(C_2H_5)_2$, $N(C_3H_7)_2$; and NH-acyl, wherein the acyl radical contains 1 to 32 carbon atoms, particularly CH_3O, preferably 9 to 32, and more preferably 9 to 20 carbon atoms;

wherein R2 is selected from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, O, and S;

wherein R3 is selected from a group consisting of: H, CH_3, and C_2H_5;

wherein R4 and R6 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, acetyl, and OX where X is a C1 to C32 acyl radical, particularly a C9 to C32 acyl radical, and preferably a C9 to C20 acyl radical;

wherein R5 is selected from a group consisting of: phenyl, CH_3, C_2H_5, C_3H_7, butyl, isobutyl, and t-butyl;

wherein R7 and R8 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, CH_3, COOH, CHO, and COOR9, wherein R9 is CH_3, C_2H_5, C_3H_7, or butyl;

wherein R10 is selected from a group consisting of: H, CH_3, and C_2H_5, and

-- represents an optional double bond;

particularly 5,6,7,8-tetrahydrobiopterin; as well as the pharmaceutically acceptable salts thereof.

In summary, it can be said that with the compounds described within the framework of the present invention, it is possible for the first time to treat certain genetic amino acid metabolic disorders by medication so that patients present improvement in protein tolerance as well as extensive normalization of their enzyme activity disorders, as well as improvements in concentrations of the affected amino acids and/or their metabolites in bodily fluids and cells.

Furthermore, the present invention proposes compositions for dietary supplements and special foods that simultaneously contain the compositions described in the invention for the improvement of protein tolerance and the decomposition of phenylalanine. It is thus possible, for the first time, to feed patients with amino acid metabolic disorders practically normally, i.e., with almost all taste and compositional nuances.

In addition to the above compounds, mentioned numerous times, the following compounds can also be used as preferred embodiments for all categories of claims:

All individual compounds as well as the various enantiomers thereof resulting from the general formulas presented using the disclosed substituents R1 through R10 and X, as well as all subcombinations thereof.

In particular, the following subcombinations of compounds are components of the disclosure:

<graphic>

wherein R1 is selected from a group consisting of: H, OH, and SH; and/or

wherein R1 is selected from the group consisting of: F, Cl, Br, and I; and/or

wherein R1 is selected from the group consisting of: NH_2, $N(CH_3)_2$, $N(C_2H_5)_2$, and $N(C_3H_7)_2$; and/or

wherein R1 is NH-acyl, wherein the acyl radical contains 1 to 32 carbon atoms, particularly CH_3O, preferably 9 to 32, and more preferably 9 to 20 carbon atoms; and/or

wherein R2 is selected from the group consisting of: H, OH, and SH; and/or

wherein R2 is selected from the group consisting of: NH_2, F, Cl, Br, I, O, and S; and/or

wherein R3 is selected from the group consisting of: H, CH_3, and C_2H_5; and/or

wherein R4 and R6 are selected independently from the group consisting of: H, OH, SH, and NH_2; and/or

wherein R4 and R6 are selected independently from the group consisting of: F, Cl, Br, and I; and/or

wherein R4 and R6 are independently acetyl; and/or

wherein R4 and R6 are selected independently from the group consisting of: OX where X is a C1 to C32 acyl radical, particularly a C9 to C32 acyl radical, and preferably a C9 to C20 acyl radical; and/or

wherein R5 is selected from the group consisting of: CH_3, C_2H_5, C_3H_7, butyl, isobutyl, and t-butyl; and/or

wherein R5 is phenyl; and/or

wherein R7 and R8 are selected independently from a group consisting of: H, OH, SH, NH_2, F, Cl, Br, I, CH_3, COOH, and CHO; and/or

wherein R7 and R8 are independently selected from the group consisting of: COOR9, wherein R9 is CH_3, C_2H_5, C_3H_7, or butyl; and/or

wherein R10 is selected from the group consisting of: H, CH_3, and C_2H_5, and -- represents an optional double bond.

It has also been shown that lipophilic tetrahydrobiopterin derivatives, such as described, for example, in EP 0 164 964 A1, are particularly suited, on the one hand, to increasing the serum half-life from approximately 8 hours to more than 18 hours in comparison to tetrahydrobiopterin. On the other hand, such lipophilic tetrahydrobiopterin derivatives are particularly suited to the production of special foods and dietary supplements since they also dissolve well in fatty mixtures, such as milk substitutes.

Moreover, the advantage of lipophilic compounds lies in their lowered sensitivity to oxidation.

Such lipophilic compounds are, in particular, those in which

R1 in the above general formula is an NH-acyl, where the acyl radical contains, in particular, 9 to 32 carbon atoms, and preferably 9 to 20 carbon atoms; and/or

R4 and R6 are selected independently from the group consisting of OX where X is, in particular, a C9 to C32 acyl radical, and preferably a C9 to C20 acyl

radical; where the substituents R2, R3, R5, R7, R8, R9, and R10 can be selected as disclosed within the framework of the present invention.

Preferably, the following tetrahydrobiopterin derivatives can be used, for example, for purposes of the present invention:

2-N-stearoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or
2-N-decanoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or
2-N-palmitoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin; and/or
2-N-linoleoyl-1',2'-di-O-acetyl-5,6,7,8-tetrahydrobiopterin.

Tetrahydrobiopterin is currently available on the market, e.g., as sapropterin hydrochloride, which is marketed under the name BIOPTEN® by the Suntory company and is used for the treatment of genetic tetrahydrobiopterin synthesis disorders.

Moreover, tetrahydrobiopterin and derivatives thereof can be produced synthetically. For example, EP 0 164 964 A1 can be mentioned here; among other things, it describes the production of a series of acylated tetrahydrobiopterin derivatives. Furthermore, US 4,665,182 discloses the organic chemical synthesis of biopterin derivatives.

The person skilled in the art will therefore not have any difficulties with the production of the compounds used.

Further advantages and characteristics are presented based on the description of embodiments and the drawings.

They show:

Drawing 1 shows phenylalanine concentrations in the blood before a challenge with phenylalanine and before and after administration of tetrahydrobiopterin in mild hyperphenylalaninemia, mild phenylketonuria, mild phenylketonuria not responding to tetrahydrobiopterin, and classic phenylketonuria;

Drawing 2 shows the effects of short-term tetrahydrobiopterin treatment on phenylalanine oxidation;

Drawing 3 shows the relation between the cumulative recovery rate of ^{13}C-marked CO_2 during administration of ^{13}C-marked phenylalanine and phenylalanine blood concentrations before and after administration of tetrahydrobiopterin;

Drawing 4 shows the effect of tetrahydrobiopterin on peripheral phenylalanine clearance and oxidation rates in patients with hyperphenylalaninemia; and

Drawing 5 shows the structural localization of phenylalanine hydroxylase missense mutations.

Table 1 shows the correlation of genotypes to clinical phenotypes.

Examples

Method of Proceeding

To explore the therapeutic efficacy of tetrahydrobiopterin, we performed a combined phenylalanine – tetrahydrobiopterin loading test for diagnostic purposes and analyzed the *in vivo* rates of [^{13}C]-phenylalanine oxidation in 38 persons with phenylalanine hydroxylase deficiency before and after administration of

tetrahydrobiopterin derivatives by assay. Responsiveness to tetrahydrobiopterin was associated with specific genotypes and we mapped mutations using a structural model of the phenylalanine hydroxylase monomer and the resultant protein misfolding.

Results

In 27 (87%) of the 31 patients with mild hyperphenylalaninemia (n = 10) or mild phenylketonuria (n = 21), tetrahydrobiopterin significantly lowered blood phenylalanine levels and enhanced/improved phenylalanine oxidation. Conversely, none of the seven patients with classic phenylketonuria (n = 7) met the criterion of strong response to tetrahydrobiopterin as defined in the study. In the case of individual patients with classic phenylketonuria, however, mild effects could be detected. Long-term treatment with tetrahydrobiopterin in five children increased daily phenylalanine tolerance significantly from 8.7 ± 8.6 mg/kg of body weight (range: 8.8 – 30) to 61.4 ± 27.9 mg/kg of body weight (range: 17.9 – 90) with drug treatment (P = 0.0043) and thus allowed them to discontinue their restricted diets. Seven mutations of the phenylalanine hydroxylase gene (P314S, Y417H, V177M, V245A, A300S, E290G, and IVS4-5C→G) and resultant structural abnormalities and misfolding of the enzyme were classified as most probably causally associated with responsiveness to tetrahydrobiopterin, and six mutations (A403V, F398L, D415N, S310Y, R158Q, and 165T) were classified as potentially associated. Four mutations (Y414C, L48S, R261Q, and 165V) were inconsistently associated with this phenotype. Mutations related to tetrahydrobiopterin responsiveness were located predominantly in the catalytic domain of the protein and were not directly involved in cofactor binding.

Conclusions:

Responsiveness to tetrahydrobiopterin derivatives – characterized by improvement in protein tolerance, extensive normalization of phenylalanine hydroxylase activity disorders and reduction of elevated phenylalanine

concentrations – frequently occurred in patients with a mild phenotype of hyperphenylalaninemia. The response can not be predicted reliably based on genotype, particularly in the case of combined double heterozygous genotypes. Drug treatment with tetrahydrobiopterin derivatives and/or the addition of the compounds to foodstuffs was able to free many patients from their very burdensome low-phenylalanine diet and thus facilitate feeding.

After this patent application was filed, the data on the invention were published and documented after peer review: New England Journal of Medicine, 2002, 347 (26), 2122-2132 (12/26/02).

Introduction

Hyperphenylalaninemia, a common inherited metabolic disorder, was one of the first genetic disorders that could be treated. In most cases, hyperphenylalaninemia results from phenylalanine hydroxylase deficiency (Ec1.14.16.1) due to mutations in the phenylalanine hydroxylase gene. The associated phenotypes range in severity from classic phenylketonuria (MIM261600) to mild phenylketonuria and mild hyperphenylalaninemia. At least half the affected patients suffer from one of the milder clinical phenotypes. Patients with classic phenylketonuria and mild phenylketonuria must follow a lifelong low-protein diet, to prevent neurological sequelae and to ensure normal

cognitive development. The highly-restrictive diet is associated with a risk of diet-related nutritional deficiencies and represents a burden for the patients and their families. Only patients with mild hyperphenylalaninemia do not require treatment, under certain circumstances. Therefore, a search for non-dietary alternative treatment methods has been encouraged.

In approximately 50 genetic diseases in humans, treatment with high doses of a cofactor can stimulate enzyme activity. Tetrahydrobiopterin is a natural cofactor of aromatic amino acid hydroxylases and nitric oxide synthase. Supplementation with this co-factor component is an established treatment method in rare cases of hyperphenylalaninemia due to inborn defects in the biosynthesis of tetrahydrobiopterin. More than 98% of patients with hyperphenylalaninemia have mutations, however, in the phenylalanine hydroxylase gene, and they have elevated rather than decreased plasmas concentration of biopterin, attributable to the action of guanosine triphosphate cyclohydrolase I feedback regulatory protein. The therapeutic use of tetrahydrobiopterin in patients with phenylalanine hydroxylase deficiency was therefore not considered.

Most recently, individual patients with mutations in the phenylalanine hydroxylase gene have been shown to have a decrease in blood phenylalanine concentrations after tetrahydrobiopterin was administered to them for diagnostic purposes. It is, however, known that peripheral phenylalanine values are governed by various genetic loci and modifying factors, and there is no evidence that the beneficial effect of tetrahydrobiopterin occurs at the level of phenylalanine hydroxylation.

This study, conducted on patients selected randomly, looks at the following questions: (1) How common is responsiveness to tetrahydrobiopterin? (2) Does tetrahydrobiopterin restore phenylalanine oxidizability? (3) Is responsiveness to tetrahydrobiopterin linked to specific genotypes and do the associated mutations map to specific points in the protein structure? (4) Does long-term treatment improve protein tolerance?

Procedure

Patients

We obtained written consent from the families of 38 children with various classes of hyperphenylalaninemia. They were classified according to plasma phenylalanine concentrations before treatment: < 600 μmol/L, mild hyperphenylalaninemia, n = 10, age 15 days to 10 years; 600 – 1200 μmol/L, mild phenylketonuria, n = 21, age 8 days to 17 years; > 1200 μmol/L, classic phenylketonuria, n = 7, age 1 day to 9 years. Defects in tetrahydrobiopterin biosynthesis or in the recycling of tetrahydrobiopterin were excluded by analysis of urinary pterin values and dihydropteridine reductase activity in erythrocytes. We analyzed 7 patients during the newborn period and 31 patients who were older. Affected siblings (n = 5) were also accepted for examination since non-genetic factors are known to influence phenylalanine homeostasis.

Combined Phenylalanine and Tetrahydrobiopterin Loading Test

Phenylalanine was ingested by having patients consumed a meal containing 100 mg of phenylalanine per kilogram of body weight. One hour after the end of the meal, the patients ingested 20 mg of tetrahydrobiopterin per kilogram (Schircks Laboratories, Jona, Switzerland). Blood phenylalanine concentrations were determined by electrospray ionization tandem mass spectroscopy before the ingestion of phenylalanine and before and after the tetrahydrobiopterin challenge (at 4, 8, and 15 hours). During the test phase, the neonates were breast fed, while the older children received a standardized protein intake (10 mg of phenylalanine per kilogram) between six and eight hours after the challenge with tetrahydrobiopterin.

In Vivo Analysis of L-Phenylalanine Oxidation

The tests were performed after a four hour fast for the younger children and an overnight fast for the older children. A total of 6 mg of L-[1-^{13}C] phenylalanine per kilogram of body weight (Euriostop, Paris, France] was given orally. The tracer was dissolved in a 25% dextrose solution (2 mg per milliliter). Breath samples were subsequently taken over a period of 180 minutes and stored in evacuated glass tubes until analysis by isotope ratio mass spectroscopy (deltaS, Thermoquest, Bremen). The recovery of carbon-13 in the breath samples was calculated as described by Treacy et al, assuming a total carbon dioxide production of 300 mmol per hour per square meter of body surface area. The $^{13}CO_2$ production was expressed as the cumulative percentage of the dose administered as a function of time. The validity of the results in neonates might be influenced by their diet or the fact that the breath sampling is more difficult than in older children. The baseline

percentage of ^{13}C, measured at time 0, did not vary significantly between the newborns and the older children, however. Values were considered to be below the detection threshold if the signal intensity of the breath % excess at time t, obtained by subtraction of the mean baseline value, did not allow sufficient distinction from atmospheric $^{13}CO_2$. On average, fewer than 1 (older child) and fewer than 2 (neonates) out of 27 consecutive $^{13}CO_2$ measurements obtained during the 180 minutes required for an individual test could not be interpreted. This had a negligible influence on the final calculation.

Mutational Analysis

DNA was extracted from leukocytes according to standard protocols. Thirteen genomic fragments covering the entire coding sequence and the exon-flanking intron sequence of the phenylalanine hydroxylase gene were amplified by polymerase chain reaction followed by direct sequencing.

Mapping of Phenylalanine Hydroxylase Gene Mutations

A model of the full-length, tetrahydrobiopterin-bound phenylalanine hydroxylase monomer was constructed from the crystal structures of several truncated forms by superimposing the catalytic domains using the tools provided by SWISS-MODEL/Swiss-Pdb Viewer.

Results

Effects of Tetrahydrobiopterin on Blood Phenylalanine Levels and Rates of Phenylalanine Oxidation

Patients were classified as responsive to tetrahydrobiopterin when blood phenylalanine levels 15 hours after tetrahydrobiopterin challenge had decreased by more than 30% from the value obtained before the administration of tetrahydrobiopterin. Tetrahydrobiopterin responsiveness was observed in all 10 patients with mild phenylalaninemia and in 17 of 21 patients with mild phenylketonuria. Only four patients with mild phenylketonuria and all seven patients with classic phenylketonuria did not fulfill the criterion of responsiveness to tetrahydrobiopterin (Drawing 1). Some patients had a rapid decrease in phenylalanine concentrations resembling those seen in patients with defects in the synthesis of tetrahydrobiopterin, whereas others had a slow response and first reached the lowest phenylalanine concentration 15 hours after the administration of the cofactor (data not shown).

Patients with varying degrees of clinical severity of the disease achieved basal cumulative recovery rates of $^{13}CO_2$ reflecting their individual residual phenylalanine oxidation capacities (classic phenylketonuria, mean value 1.4%; mild phenylketonuria, 3.1%; mild hyperphenylalaninemia, 5.6% healthy controls, 9.0%). During treatment with tetrahydrobiopterin (10 mg/kg of body weight over 24 hours), the cumulative recovery of $^{13}CO_2$ significantly increased in the same groups that had had a response to the loading test. The increase was more pronounced in those with mild phenylketonuria than in those with mild

hyperphenylalaninemia (Drawing 2A). It is noteworthy that 8 out of 11 patients who did not respond showed a slight increase in phenylalanine oxidation after short-term treatment with tetrahydrobiopterin; in three of these patients, the blood phenylalanine concentration was affected simultaneously. This suggests that, with longer-term therapy, minor improvements can be achieved with tetrahydrobiopterin derivatives even in more severe forms of hyperphenylalaninemia. The time curves of the fractional $^{13}CO_2$ formation deviated markedly from that of the normal-oxidation phenotype (Drawings 2B, C, D and E). With cofactor treatment, the curves reverted toward normal in patients who had a response to tetrahydrobiopterin (Drawings 2B and C), but remained unchanged in patients who did not have a response to tetrahydrobiopterin

Before tetrahydrobiopterin treatment, all patients had blood phenylalanine concentrations above 200 µmol/L and cumulative rates of recovery of $^{13}CO_2$ below 7%, with considerable overlap between patients with a response and those without a response. After tetrahydrobiopterin treatment, the two patient groups formed two non-overlapping clusters. Among the tetrahydrobiopterin-sensitive patients were 4 children who exhibited a moderate response to tetrahydrobiopterin (Drawing 3).

The degree of intersubject variability was large: tetrahydrobiopterin challenge reduced phenylalanine levels by 37 to 92% when blood values were compared before and 15 hours after the administration of tetrahydrobiopterin. In 23 of 27 patients with a response to tetrahydrobiopterin, blood phenylalanine concentrations decreased below 200 µmol/L, whereas in 4 patients responded with values between 200 and 400 µmol/L. In patients with no response, blood phenylalanine concentrations always exceeded 400 µmol/L after the

tetrahydrobiopterin challenge. Tetrahydrobiopterin enhanced the oxidation rates of ^{13}C phenylalanine by 10 to 91% and resulted in oxidation rates within the normal range in 22 of the 27 patients with a response to tetrahydrobiopterin. The remaining five patients had an improvement, but the rates did not reach the normal range. Although generally consistent, some patients presented with noteworthy irregularities in the tetrahydrobiopterin effect at the two endpoints analyzed (examples given in Drawing 4). One patient with classic phenylketonuria had a slight increase in blood phenylalanine concentrations as well as an improvement in phenylalanine oxidation rate, but did not meet the criterion for strong responsiveness of tetrahydrobiopterin (Drawing 4).

Long-Term Treatment with Tetrahydrobiopterin

The families of five children with mild phenylketonuria (age, 4 to 14 years) agreed to a therapeutic trial replacing dietary phenylalanine restriction with the oral administration of tetrahydrobiopterin at daily doses of 7.1 to 10.7 mg/kg of body weight. The treatment lasted for 207.0 ± 51.3 days (mean ± SD, range 166 to 263). The cofactor treatment led to an increase in daily phenylalanine tolerance, from 8.7 ± 8.6 mg/kg of body weight (range, 8.8 - 30.0) before treatment to 61.4 ± 27.9 mg/kg of body weight (range, 17.9 - 90.0) during treatment ($P = 0.0043$), with little effect on the blood concentrations of phenylalanine (366 ± 120 µmol/L during dietary treatment and 378 ± 173 µmol/L during exclusive cofactor treatment).

Identification and Mapping of Phenylalanine Hydroxylase Gene Mutations

In 37 of 38 patients, two mutant alleles were identified (Table 1). We classified seven mutations (P314S, Y417H, V177M, V245A, A300S, E390G, and IVS4–5C>G) as *most probably* responsible for responsiveness to tetrahydrobiopterin, because they were present in either the homozygous or a functional hemizygous state. Six additional mutations were *potentially* connected to tetrahydrobiopterin responsiveness because of considerable residual *in vitro* enzyme activity (A403V, F39L, D415N, R158Q, and I65T), as previously described, or because of a known severe mutation on the second allele (S310Y). Four mutations (Y414C, L48S, R261Q, and I65V) were *inconsistently* associated with tetrahydrobiopterin responsiveness. Eight of 12 missense mutations connected to tetrahydrobiopterin responsiveness mapped to the catalytic domain, whereas 2 mapped to the regulatory domain and 2 to the tetramerization domain. None of them affected residues at the active site or amino acids that interacted directly with the cofactor (Drawing. 5).

Discussion

We present several lines of evidence that the metabolic phenotype of phenylalanine hydroxylase deficiency can be modified by pharmacologic doses of tetrahydrobiopterin or derivatives thereof. First, tetrahydrobiopterin loading led to normal or nearly normal blood phenylalanine concentrations in most patients with residual phenylalanine hydroxylase enzyme activity, suggesting that responsiveness to tetrahydrobiopterin is a common feature of mild

hyperphenylalaninemia phenotypes. Second, tetrahydrobiopterin enhanced residual phenylalanine oxidative capacity in these patient groups. Third, long-term tetrahydrobiopterin treatment led to a significant improvement in protein tolerance and eliminated the need for an overly restrictive diet.

Our findings suggest that the *in vitro* phenylalanine oxidation test can discriminate among classes of hyperphenylalaninemia of different severity. This observation is in accordance with data on the ability of the method to measure the dose effects of the phenylalanine hydroxylase gene. However, because of the multifactorial nature of hyperphenylalaninemia, the whole-body rate of phenylalanine oxidation is not a simple equivalent of phenylalanine hydroxylase activity. The decrease in blood phenylalanine concentrations was accompanied by a significant increase in *in vivo* phenylalanine oxidative capacity in all patients who were identified as responsive to tetrahydrobiopterin. Taken together, these observations are consistent with the hypothesis that the misfolding of the enzymes and impaired phenylalanine hydroxylation is improved by tetrahydrobiopterin therapy. The extent of the improvement in the disposal of phenylalanine did not always correspond to the change in phenylalanine oxidation, a finding not unexpected with respect to genetically determined enzyme deficiencies in general and phenylalanine hydroxylase deficiency in particular. We observed slow and rapid responses as well as differences in the time course and relative extent of $^{13}CO_2$ formation, suggesting that tetrahydrobiopterin may exert its effects through various mechanisms and with

different degrees of efficacy, depending on the extent of the protein misfolding. In addition to the proposal that high-dose tetrahydrobiopterin treatment may compensate for the decreased affinity of the defective phenylalanine hydroxylase for tetrahydrobiopterin, other mechanisms need to be considered.

Tetrahydrobiopterin treatment may up-regulate the expression of the phenylalanine hydroxylase gene, stabilize phenylalanine hydroxylase messenger RNA, facilitate the formation of functional phenylalanine hydroxylase tetramers, or protect a misfolded enzyme protein from proteolytic cleavage.

The use of genotyping to predict the phenotype may present difficulties in the case of complex diseases due to multiple genetic factors, such as hyperphenylalaninemia. We identified predominantly "mild" genotypes in the group of patients with a response to tetrahydrobiopterin, whereas most of the patients without a response had "severe" genotypes. The weight of the evidence of the association of distinct mutations with responsiveness to tetrahydrobiopterin varied, and predictions based on genotypes are therefore difficult, particularly in the presence of compound heterozygotes. The Y414C mutation is known to occur in more than one clinical phenotype. We have identified this mutation in a functional hemizygous state in two patients with identical genotypes but discordant responses to tetrahydrobiopterin. This observation may be explained by the influences of modifying loci in hyperphenylalaninemia. In a homozygous state, and thus one in which homopolymeric tetramers are formed, the Y414C and the L48S mutations were reported to confer responsiveness to tetrahydrobiopterin. However, we detected these mutations in a functional hemizygous state in patients with classic phenylketonuria who had no response to tetrahydrobiopterin. Under these conditions, heteropolymerization may impede the formation of functional tetramers.

Our data confirm the assumption that most missense mutations associated with sensitivity to tetrahydrobiopterin are in the catalytic domain of the protein, but

they do not map to residues at the active site and are not directly involved in cofactor binding. These mutations may affect interactions between domains in a monomer or influence residues in the dimer or tetramer interfaces, resulting in the misfolding of the protein and reduced enzyme activity. Tetrahydrobiopterin therefore serves as a chemical chaperone and prevents this.

Previously, *in vitro* expression analysis has been used to predict the functional effect *in vivo* of mutations in the phenylalanine hydroxylase gene. This type of analysis may result in the overestimation of phenylalanine hydroxylase activity *in vitro* compared to *in vivo*. This may be explained by the fact that such analyses have been carried out almost exclusively in the presence of high concentrations of natural or synthetic cofactors, which makes genotype–phenotype correlation more difficult. Revised experimental protocols to assess the intrinsic severity of mutations should include a range of tetrahydrobiopterin concentrations.

Since responsiveness to tetrahydrobiopterin cannot be predicted on the basis of pretreatment phenylalanine concentrations, we would suggest a new clinical classification: (1) *tetrahydrobiopterin-unresponsive hyperphenylalaninemia;* and (2) *tetrahydrobiopterin-responsive hyperphenylalaninemia*, which includes (a) tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency and (b) defects in the synthesis of tetrahydrobiopterin. A phenylalanine–tetrahydrobiopterin loading test with an extended observation period (≥15 hours) can reliably and safely discriminate

between patients with a response and patients without a response and ought to be performed in all patients with hyperphenylalaninemia to identify those who may benefit from tetrahydrobiopterin treatment. Our short-term study design does not exclude the possibility of subtle effects, which may become evident only after prolonged treatment even in some patients with classic phenylketonuria.

Our data suggest that long-term therapy with tetrahydrobiopterin could lead to an increase in phenylalanine tolerance. Cofactor treatment instead of a burdensome restricted diet is suitable many patients and treatment with tetrahydrobiopterin derivatives would be expected to improve their quality of life substantially. In particular, the addition of these compounds to goods would considerably ease the creation of an otherwise very difficult diet. Tetrahydrobiopterin treatment may also be helpful in cases of maternal phenylketonuria, since strict metabolic control, which is key to the prevention of serious adverse effects in the offspring, is difficult to maintain during pregnancy. However, the safety or lack of side effects of tetrahydrobiopterin therapy during pregnancy has not been established. Worldwide, more than 350 patients with tetrahydrobiopterin deficiency have been treated with the cofactor. Some dose-dependent adverse reactions, including sleep disorders, polyuria, and loose stools, were reported in a safety evaluation (BIOPTEN® approval letter, Suntory, Japan).

Several obstacles must be overcome before tetrahydrobiopterin treatment can be used routinely. First, tetrahydrobiopterin has not yet been approved for therapeutic use in most countries. Second, this compound is expensive. Third, dose-finding studies and clinical trials are needed to determine the bioavailability

and long-term effects of tetrahydrobiopterin therapy in patients with phenylalanine hydroxylase deficiency.

In summary, we have shown that pharmacologic doses of tetrahydrobiopterin considerably improved impaired phenylalanine oxidation in the majority of patients with a less severe hyperphenylalaninemia phenotype through elimination of protein misfolding. Furthermore, an improved protein tolerance and an easing of dietary restrictions can be achieved. These results are important to the diagnostic workup, clinical classification and therapeutic treatment of this defect. In the near future, in a large number of patients, cofactor treatment may obviate the need for the most burdensome dietary restrictions.

TABLE 1. GENOTYPES IN 38 PATIENTS WITH TETRAHYDROBIOPTERIN-SENSITIVE AND NONSENSITIVE HYPERPHENYLALANINEMIA

ID	ALLELE 1	ALLELE 2	PHENOTYPE	TETRAHYDROBIOPTERIN SENSITIVITY
1	A403V	IVS4+5G>T	Mild	Yes
2	A403V	Not identified	Mild	Yes
3	P314S*	R408W†	Mild	Yes
4	F39L	D415N	Mild	Yes
5	**Y414C**	D415N	Mild	Yes
6	Y417H*	Y417H*	Mild	Yes
7	F55L	S310Y*	Mild Phenylketonuria	Yes
8	*R261Q*	**Y414C**	Mild	Yes
9	V177M	R408W†	Mild	Yes
10	P275L*	**Y414C**	Mild Phenylketonuria	Yes
11	V245A	R408W†	Mild	Yes
12	**L48S**	R158Q	Mild Phenylketonuria	Yes
13	Y417H*	Y417H*	Mild Phenylketonuria	Yes
14	V245A	R408W†	Mild	Yes
15	R261X†	A300S	Mild Phenylketonuria	Yes
16	R158Q	E390G	Mild Phenylketonuria	Yes
17	R261X†	A300S	Mild Phenylketonuria	Yes
18	*Y414C*	IVS12+1G>A†	Mild Phenylketonuria	Yes
19	I65S*	A300S	Mild Phenylketonuria	Yes
20	*R261Q*	*Y414C*	Mild Phenylketonuria	Yes
21	K274fsdel11b	E390G	Mild Phenylketonuria	Yes
22	IVS4-5C>G	R408W†	Mild Phenylketonuria	Yes
23	R261X†	A300S	Mild Phenylketonuria	Yes
24	I65T	**Y414C**	Mild Phenylketonuria	Moderate
25	E390G	IVS12+1G>A†	Mild Phenylketonuria	Moderate
26	I65V	*R261Q*	Mild	Moderate
27	R158Q	**Y414C**	Mild Phenylketonuria	Moderate

Table 1 – Continued

ID	ALLELE 1	ALLELE 2	PHENOTYPE	TETRAHYDROBIOPTERIN SENSITIVITY
28	**Y414C**	IVS12+1G>A†	Classic	No
29	P281L†	**Y414C**	Mild Phenylketonuria	No
30	I65V	IVS12+1G>A†	Mild Phenylketonuria	No
31	**I65V**	IVS12+1G>A†	Mild Phenylketonuria	No
32	N61D*	*R261Q*	Mild Phenylketonuria	No
33	R408W†, R413P	**Y414C**	Classic	No
34	P281L†	P281L†	Classic	No
35	R243X†	**Y414C**	Classic	No
36	L48S	P281L†	Classic	No
37	*R261Q*	R408W†	Classic	No
38	R243X†	IVS7+1G>A	Classic	No

Mutations potentially associated with sensitivity to tetrahydrobiopterin are printed in gray.
Mutations potentially associated with sensitivity to tetrahydrobiopterin are printed in bold.
Mutations inconsistently associated with sensitivity to tetrahydrobiopterin are printed in italics.

* Mutation not described previously

† Putative null mutation.

N.I. Not identified

Key to the Drawings

Drawing 1

Effect of Tetrahydrobiopterin on Blood Phenylalanine Concentrations.
Blood Phenylalanine Concentrations before Phenylalanine Loading (Phe) and before and after Challenge with Tetrahydrobiopterin (BH4). The boxes show the 50% confidence interval (25th - 75th percentiles), the horizontal black bars represent the medians, and the I bars indicate the range between minimum and maximum. The P value refers to the difference between the blood phenylalanine concentration before and 15 hours after the administration of tetrahydrobiopterin.

Drawing 2

Effect of Short-Term Treatment with Tetrahydrobiopterin on Phenylalanine Oxidation *In Vivo.* **A** shows the cumulative recovery of $^{13}CO_2$ (180 minutes) before and after tetrahydrobiopterin treatment (BH_4). The boxes show the 50% confidence interval (25th - 75th percentile), the horizontal black bars represent the medians, and the I bars indicate the range between minimum and maximum. **B – E:** Fractional analysis of $^{13}CO_2$ formation rate in four representative patients with impaired phenylalanine hydroxylase activity before (□) and after (λ) short-term treatment with tetrahydrobiopterin.

Drawing 3

Relation between the Cumulative Recovery of $^{13}CO_2$ (180 Minutes) and the Blood Phenylalanine Concentration Before and After the Administration of

Tetrahydrobiopterin (BH4). Patients not sensitive to tetrahydrobiopterin O; Patients sensitive to tetrahydrobiopterin λ; Patients moderately sensitive to tetrahydrobiopterin λ.

Drawing 4

Effect of Tetrahydrobiopterin on Peripheral Phenylalanine Clearance and Oxidation Rates in Individual Patients with Hyperphenylalaninemia. The blood phenylalanine concentrations before (solid bars) and 15 hours after the administration of tetrahydrobiopterin (BH_4) (dark gray bars). The positive effects obtained in individual patients with tetrahydrobiopterin are shown by a black arrow (upper graph). Cumulative recovery of $^{13}CO_2$ (180 minutes) before (light gray bars) and after the ingestion of tetrahydrobiopterin (solid bars). The improvement caused by tetrahydrobiopterin in individual patients is shown by a black arrow (lower graph). The normal range (n.r.) of *in vivo* phenylalanine oxidation in the healthy controls (age range, 2 days to 13 years) is shown (8.3 ± 2.8% mean ± S.D., n = 12). Imbalance in the effect of tetrahydrobiopterin: distinct decrease in blood phenylalanine concentrations, but little enhancement in phenylalanine oxidation in one patient (λ) and little effect on blood phenylalanine concentration and large increase in phenylalanine oxidation in another patient (H). Slight response to tetrahydrobiopterin does not meet the criterion of tetrahydrobiopterin responsiveness in one patient with classic phenylketonuria (ν).

Drawing 5

Structural Localization of Phenylalanine Hydroxylase Missense Mutations. The phenylalanine hydroxylase monomer, shown as a ribbon, is composed of three functional domains: the regulatory domain (residues 1 to 142), the catalytic domain (residues 143 to 410), and the tetramerization domain (residues 411 to 452). The active-site iron (partially obscured brown sphere) and the cofactor analogue 7,8-dihydro-tetrahydrobiopterin stick model are in the catalytic domain. Mutations that are *most probably* associated with responsiveness to tetrahydrobiopterin are turquoise. Mutations that are *potentially* associated with responsiveness to tetrahydrobiopterin are green. Mutations that are *inconsistently* associated with responsiveness to tetrahydrobiopterin are magenta.